Original Article

MyD88 deficiency aggravates the severity of acute pancreatitis by promoting MyD88-independent TRIF pathway-mediated necrosis

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Background: With uncontrolled inflammatory progression, acute pancreatitis (AP) can progress to severe acute pancreatitis (SAP). Inflammation and parenchymal cell death are key pathologic responses of AP. Toll-like receptor 4 (TLR4) plays a pro-inflammatory role in AP. Myeloid differentiation primary response protein 88 (MyD88) is the most essential utilized adaptor of TLR4, but its role in AP remains unclear. We investigated the potential role of MyD88 in the pathogenesis of AP.

Methods: An AP model was induced by administering either cerulein or L-arginine to wild-type or MyD88-deficient mice. Additionally, receptor-interacting protein kinase 1 (RIP1) inhibitor necrostatin-1 (Nec-1) was administered to the MyD88$^{-/-}$ mice. The severity of AP was determined by measuring serum amylase and lipase activities, quantifying pancreatic myeloperoxidase (MPO) activity, and histological examination. The effects of MyD88 deletion on cell death and the inflammatory response were determined by measuring apoptosis, necrosis, and inflammatory cytokines. Western blot was used to assess the necrotic mediators, RIP1 and RIP3.

Results: The deletion of MyD88 resulted in more severe acute experimental pancreatitis as assessed by increased amylase and lipase activities, increased pancreatic MPO activity, a reduced anti-inflammatory response, reduced apoptosis, and increased necrosis. Additionally, Nec-1 treatment significantly reduced necrosis in the MyD88$^{-/-}$ mice.

Conclusions: The deletion of MyD88 inhibited the TLR4/MyD88-dependent pathway mediated protective immune defense response and enhanced TLR4/MyD88-independent TRIF pathway-mediated pancreatic necrosis, which in turn aggravated the severity of AP. The critical role of MyD88 in immune defense response and cell death indicates that MyD88 represents a potential therapeutic target in the management of AP.

Keywords: Acute pancreatitis; myeloid differentiation primary response protein 88 (MyD88); Toll-interleukin receptor domain-containing adaptor-inducing interferon-β (TRIF); receptor-interacting protein kinase; inflammation

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Introduction

Acute pancreatitis (AP) is an inflammatory disease with an increasing incidence rate worldwide (1). Severe acute pancreatitis (SAP) has a high mortality rate of up to 40% (2). During AP, a key initiating event is premature activation of zymogens, resulting in pancreatic acinar cells damage. These damaged pancreatic acinar cells release pro-inflammatory mediators and recruit inflammatory cells (3). In the progression of AP, inflammation and parenchymal cell death are 2 pivotal pathologic responses that determine the severity of the AP (3). Necrosis and apoptosis are the main forms of parenchymal cell death, and the severity of AP has been shown to be directly correlated with necrosis and inversely correlated with apoptosis (4-7).

Apoptosis is a highly coordinated process that may be activated by intrinsic and extrinsic pathways. It is executed by the activation of caspases (8). Conversely, necrosis is dependent on the activities of receptor-interacting protein kinase 1 (RIP1) and RIP3, which form a pronecrotic complex with a homotypic interaction motif upon the activation of the tumor necrosis factor (TNF) receptor and related receptors (9). It has been reported that necrosis/necroptosis plays a prominent role in the pathological progress of AP. The mechanisms by which pancreatitis are initiated are not clear; however, it is thought that the disease originates from injury to the acinar cells, which leads to uncontrolled inflammation, which in turn, and more importantly, contributes to parenchymal necrosis. At present, there is no specific medical therapy targeting the inflammatory storm of AP that has been proven to have any clinical benefit (2). The discovery of new therapeutic targets requires a better understanding of the regulatory inflammatory pathways in AP.

Toll-like receptor 4 (TLR4) is one of the best-known toll-like receptors (TLRs) that respond to both pathogen-associated molecular patterns and tissue damage-related signals (10,11). TLR4 activation can also drive inflammation. Recent research on AP has shown that a lack of TLR4 ameliorates pancreatic inflammation in mice (12,13). TLR4 activates 2 distinct intracellular signaling pathways via the adaptor molecules myeloid differentiation primary response protein 88 pathway (the MyD88-dependent pathway) and the Toll-interleukin receptor domain-containing adaptor-inducing interferon-β (TRIF; the MyD88-independent pathway) (14). MyD88-dependent signaling triggers the classical inflammatory cascade, leading to the activation of nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPKs), which in turn lead to the production of pro-inflammatory and anti-inflammatory cytokines. MyD88 can also mediate apoptosis, and mostly does so indirectly through the production of pro-apoptotic intermediates, such as TNF-α (15). Conversely, the MyD88-independent TRIF signaling pathway leads to the activation of interferon regulatory factor 3 (IRF3) and interferon β (IFN-β) production, can mediate delayed NF-κB and MAPK activation (16), and has also been implicated in the RIP1/RIP3-mediated necrosis signaling pathways (9,17).

Based on these findings, we postulated that the Myd88-dependent and Myd88-independent signaling pathways play a role in the pathogenesis of AP. We found that the deletion of MyD88 inhibits the MyD88-dependent pathway-mediated protective immune defense response and enhances the MyD88-independent TRIF pathway, which, surprisingly, increases the severity of experimental AP mainly by promoting pancreatic necrosis via the MyD88-independent TRIF-mediated necrotic signaling pathway. Our findings improve understandings of the complex role of MyD88 in the TLR4-mediated immune defense response and cell death in AP, and may lead to innovative therapies in the treatment of SAP. We present the following article in accordance with the ARRIVE reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-5134/rc).

Methods

Animals and reagents

Wild-type C57BL/6 mice and MyD88-deficient mice were obtained from the Model Animal Research Center of Nanjing University (Nanjing, China). All the mice were maintained on a 12-h light/dark cycle with free access to water and food and acclimatized for at least 1 week. Simple random sampling was used to allocate the mice to the control and experimental groups. The mice were fasted for 18 h and only provided water *ad libitum* before the experiment. The genotype of MyD88-deficient mice was validated before each experiment.

Experiments were performed under a project license (2021737A) granted by Ethics Committee of West China Hospital Sichuan University, in compliance with the institutional guidelines for the care and use of animals. The protocol was prepared before the study, and it was not registered anywhere.

Cerulein and L-arginine hydrochloride were purchased from Sigma Chemical (Sigma, St. Louis, Missouri, USA). Antibodies against RIP1, RIP3, and β-actin were purchased from Cell Signaling Technology (CST, Danvers, Massachusetts, USA). Other items were purchased from standard suppliers or as indicated in the text.

Induction of AP

A cerulein pancreatitis model was induced in the mice (weight: 20–25 g) by 7-hourly intraperitoneal (IP) injections of 50 μg/kg of cerulein (Sigma) in saline, while the control mice were given saline as previously described (18). In addition, 1.65 mg/kg of RIP1 inhibitor necrostatin-1 (Nec-1; Santa Cruz Biotechnology, California, USA) was injected intraperitoneally 10 min before the cerulein injection, every 2 h later for 6 h in total; one-fourth of the above-mentioned concentration was applied to the $MyD88^{-/-}$ mice. The mice were killed 0, 8, and 24 h after the first injection of cerulein. For L-arginine induced pancreatitis, L-arginine hydrochloride (8%; Sigma) was prepared (pH: 7.0). The mice (weight: 20–25 g) received IP injections of L-arginine (4.0 g/kg) every 2 h, and the control mice received IP injections of saline as previously describe (19). The mice were killed at 0, 48, and 72 h after the first injection of L-arginine. The blood samples were centrifuged at 2,000 ×g for 20 min, and the serum was stored at –80 ℃. The pancreas was removed, frozen in liquid nitrogen, and stored at –80 ℃. There were 10 mice in each group, and the experiments were repeated 3 times to confirm the results. Pathological evaluation was done by two pathologists.

Biochemical assays

The levels of serum amylase and lipase were measured by means of a commercially available kit (R&D System, MN, USA). The extent of neutrophil infiltration in the pancreas was quantitated by measuring tissue myeloperoxidase (MPO) activity (20). The enzyme activity was measured by a MPO kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), and calculated as a percentage of the control.

Morphology assays

For the light microscopy, the pancreas was fixed in 4% paraformaldehyde, embedded in paraffin, and 5-mm sections were processed for hematoxylin and eosin (H&E) staining. The extent of necrosis was scored by 2 experienced morphologists.

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labelling (TUNEL) assays

TUNEL assays were used to quantify the apoptosis of the pancreatic tissue sections. The sections were stained using TUNEL (Merck & Co., Inc., USA). Dark brown staining of the nucleus was observed in TUNEL-positive cells [containing labelled deoxyribonucleic acid (DNA) fragments]. The sections were counterstained with 0.3% methyl green. The numbers of positive apoptotic cells were counted in 10 high-power fields (×400 magnification) (21).

Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)

The ribonucleic acids (RNAs) from the pancreas were prepared according to method described in Griffin's study (22). Each tissue was lysed with TRIzol (Invitrogen, California, USA) for total RNA isolation. Next, a DNA reverse-transcription system (Invitrogen) was used for the reverse transcription. Polymerase chain reaction (PCR) was performed in the presence of specific primers. The following primers were used: TLR4, forward: 5'-CAGAACTTCAGTGGCTGGA-3', reverse: 5'-TAGGGTTTCCTGTCAGTATC-3'; interleukin (IL)-1 receptor-associated kinase 4 (IRAK4), forward: 5'-CGGCGACGACAGATACAAT'-3', reverse: 5'-CTTTTGACGGTTTGGGGAA-3'; TRIF, forward: 5'-CAGGGACCGGGAGATCTA-3', reverse: 5'-CATGTTCCGAACCGACAGC-3'; TNF-α, forward: 5'-AGACCCTCACACTCAGATCAT-3', reverse: 5'-AGTCACAGAAGGAGTGGCTAA-3'; IL-6, forward: 5'-CACTAGGTTTGCCGAGTAGA-3', reverse: 5'-CGTGAAAAGATGACCCAGAT-3'; IL-10, forward: 5'-CAACATACTGCTAACCGACTC-3', reverse: 5'-CCTGGGGCATCACTTCTAC-3'; β-actin, forward: 5'-CGTGAAAAGATGACCCAGAT-3', reverse: 5'-ACCCTCATAGATGGGCACA-3'. The 40-cycle program (94 ℃ for 20 s, 53 ℃ for 30 s, and 72 ℃ for 30 s) *conditions were* used for all PCRs on an iCycler IQ (Bio-Rad) system. β-actin was used as the internal control in each reaction, and relative quantitative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (23). Each sample was

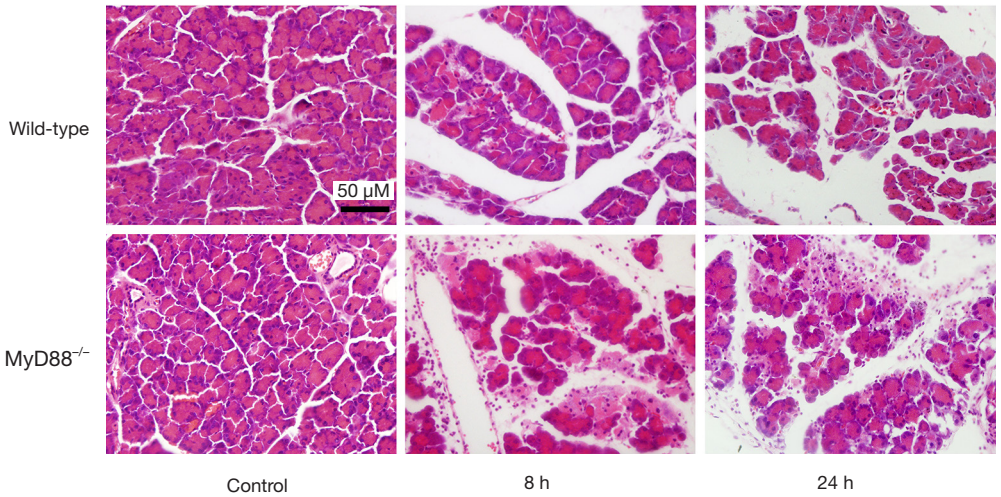

Figure 1 MyD88 deletion changes the morphology of cerulein-induced pancreatitis. Representative micrographs of H&E-stained pancreas tissue. The results revealed a significant increase in necrosis and inflammation in pancreatic tissue in the MyD88$^{-/-}$ mice induced by cerulein compared to the wild-type mice. MyD88, myeloid differentiation primary response protein 88; H&E, hematoxylin and eosin.

analyzed in triplicate.

Western blot analysis

The total protein of the pancreas tissues was extracted. First, portions of the frozen pancreas tissue were rapidly homogenized in liquid nitrogen, and then reconstituted in ice-cold radioimmunoprecipitation assay buffer containing 1 mmol/L of phenylmethanesulfonylfluoride and a cocktail of protease inhibitors (1:100 dilution) (Sigma, St. Louis, Missouri, USA). The tissue lysates were rotated for 40 min at 4 ℃ and centrifuged at 4 ℃ for 15 min at 16,000 ×g. The supernatants were recovered, and total amounts of protein were measured using the bicinchoninic acid assay method (Pierce, Rockford, Illinois, USA). Twenty micrograms of protein was subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Then, transferred onto polyvinylidene difluoride membranes (Millipore, Massachusetts, USA). The membranes blocked with 5% milk powder solution for 1 h at room temperature and incubated at 4 ℃ overnight with rabbit monoclonal anti-RIP1 antibody (1:1,000 dilution), anti-RIP3 antibody (1:1,000 dilution) and anti-β-actin antibody (1:1,000 dilution). Membranes were washed for 10 min 3 times. Then, it incubated with goat polyclonal anti-rabbit immunoglobulin G secondary antibody (Cell Signaling) conjugated to horseradish peroxidase (1:5,000 dilution) for 1 h at room temperature. Finally, the antibody binding was visualized using the enhanced chemiluminescence system (Pierce, Rockford, USA).

Statistical analysis

The highest and lowest values of each group's results were removed, and the rest of the values were averaged. The results are expressed as the mean ± standard error of the mean (SEM). The data were analyzed using a one-way analysis of variance with the Tukey-Kramer *post-hoc* test. A P value <0.05 was considered statistically significant.

Results

Effect of MyD88 deletion on cerulein-induced AP

We assessed the severity of AP in the cerulein-administered wild-type and MyD88$^{-/-}$ mice. The morphological changes observed in pancreatitis included acinar cell vacuolization, edema, and necrosis, all of which were significantly more severe in the MyD88$^{-/-}$ mice than the wild-type mice (*Figure 1*). The levels of serum amylase (*Figure 2A*) and lipase (*Figure 2B*) of the MyD88$^{-/-}$ mice were significantly higher than those of the wild-type mice. Compared to the wild-type mice, the MyD88$^{-/-}$ mice had increased pancreatic

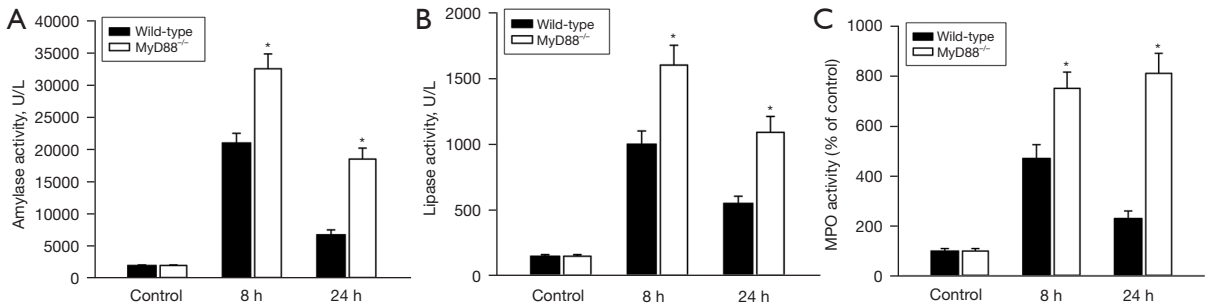

Figure 2 MyD88 deletion changes the severity of cerulein-induced pancreatitis. Serum amylase (A), lipase (B), and pancreatic MPO (C) were significantly higher in the MyD88$^{-/-}$ mice than the wild-type mice. The data are shown as the mean ± SEM. * P<0.05, *vs.* wild-type. MyD88, myeloid differentiation primary response protein 88; MPO, myeloperoxidase; SEM, standard error of the mean.

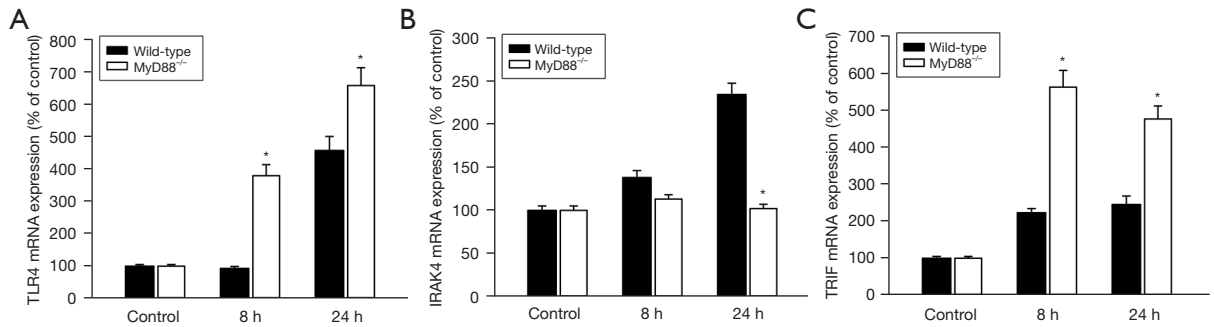

Figure 3 Effects of MyD88 deletion on expressions of TLR4, IRAK4, and TRIF in cerulein-induced pancreatitis. The expressions of TLR4 (A), IRAK4 (B), and TRIF (C) were determined by real-time RT-PCR. The results showed that compared to the wild-type mice, there was a significant increase in the TLR4 and TRIF levels of the MyD88$^{-/-}$ mice, while IRAK4 was not activated and remained at the base line level in the MyD88$^{-/-}$ mice. The data are shown as the mean ± SEM. *, P<0.05, *vs.* wild-type. MyD88, myeloid differentiation primary response protein 88; TLR4, Toll-like receptor 4; IRAK4, interleukin-1 receptor-associated kinase 4; TRIF, Toll-interleukin receptor domain-containing adaptor-inducing interferon-β; RT-PCR, reverse transcription-polymerase chain reaction; SEM, standard error of the mean.

tissue MPO activity (*Figure 2C*).

Effect of MyD88 deletion on the TLR4-mediated MyD88-dependent and MyD88-independent pathways in cerulein-induced AP

We assessed TLR4 expression in the cerulein-induced wild-type and MyD88$^{-/-}$mice. The results revealed an increase in TLR4 messenger RNA (mRNA) expression in the cerulein-treated wild-type mice compared to the control animals, and this increase was significantly more enhanced in the MyD88$^{-/-}$ mice than the wild-type mice (*Figure 3A*). We then assessed the expression of interleukin-1 receptor-associated kinase 4 (IRAK4), which is the downstream molecule of MyD88, and found that IRAK4 expression in the MyD88$^{-/-}$ mice did not change after cerulein treatment, but its expression in the wild-type mice increased 24 h after cerulein treatment compared to that of the control mice (*Figure 3B*). We also measured the expression of TRIF, which represents the MyD88-independent pathway, and noted a slight increase in TRIF mRNA expression in the cerulein-treated wild-type mice. The MyD88$^{-/-}$ mice were significantly more enhanced than the wild-type mice (*Figure 3C*). These findings indicate that the activation of IRAK4 was dependent on MyD88, and the MyD88-dependent pathway was blocked in the cerulein-induced MyD88$^{-/-}$ mice. Conversely, the TLR4-mediated MyD88-independent TRIF pathway was significantly enhanced in

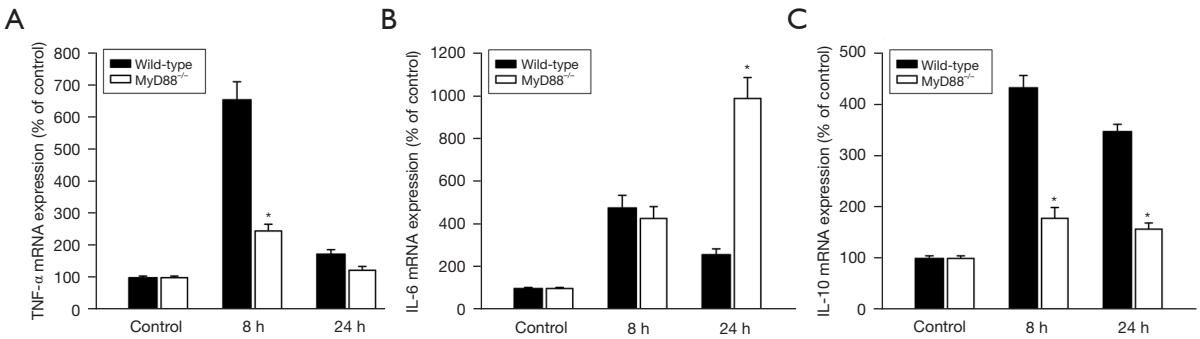

Figure 4 Effects of MyD88 deletion on inflammatory cytokines in cerulein-induced pancreatitis. The levels of TNF-α (A) and IL-6 (B), and IL-10 (C) were determined by RT-PCR. The results showed that the TNF-α and IL-10 levels were significantly reduced in the pancreatic tissue of the MyD88$^{-/-}$ mice, while the IL-6 level was significantly increased at the late stage after cerulein treatment in the MyD88$^{-/-}$ mice compared to the wild-type mice. The data are shown as the mean ± SEM. *, P<0.05, *vs.* wild-type. MyD88, myeloid differentiation primary response protein 88; TNF-α, tumor necrosis factor α; IL, interleukin; RT-PCR, reverse transcription-polymerase chain reaction; SEM, standard error of the mean.

the cerulein-induced MyD88$^{-/-}$ mice.

Effect of MyD88 deletion on the inflammatory response in cerulein-induced AP

To assess the pancreatic inflammatory response, TNF-α, IL-6, and IL-10 levels were examined by real-time RT-PCR. Both pro-inflammatory (TNF-α and IL-6) and anti-inflammatory (IL-10) cytokines were increased in the wild-type mice after cerulein treatment. TNF-α and IL-10 mRNA levels were more decreased in the MyD88$^{-/-}$ mice than the wild-type mice (*Figure 4A*,*4B*). However, at the late stage (24 h), the IL-6 mRNA level of the MyD88$^{-/-}$ mice was significantly more increased than that of the wild-type mice (*Figure 4C*).

Effect of MyD88 deletion on cell death in cerulein-induced AP

We also investigated apoptosis during cerulein-induced AP (*Figure 5A*). The *in-situ* TUNEL assays showed that apoptosis was more increased in the wild-type mice treated with cerulein at the early stage (8 h) than the control mice. Additionally, few apoptosis positive cells were detected at the late stage (24 h) of the disease. However, the increase of apoptosis in the MyD88$^{-/-}$ mice was less than that in the wild-type mice at the early stage (*Figure 5B*). A quantitative analysis of acinar cell necrosis was undertaken by standard histological examination. The results revealed that there was significantly more necrosis in the MyD88$^{-/-}$ mice than the wild-type mice (*Figure 5C*). We then assessed the necrotic mediators of RIP1 and RIP3 using Western blot assays (*Figure 6A*). RIP1 revealed a marked degradation at the early stage (8 h), and RIP3 was slightly more increased in the cerulein-induced wild-type mice than the control mice (*Figure 6B*,*6C*). Conversely, the expression of RIP1 and RIP3 was significantly more enhanced in the MyD88$^{-/-}$ mice than the wild-type mice (*Figure 6B*,*6C*).

Effect of Nec-1 on cerulein-induced necrosis in MyD88$^{-/-}$ mice

To examine the role of the RIP1 inhibitor Nec-1 on necrosis in AP in the MyD88$^{-/-}$ mice, we investigated the necrosis in the cerulein-induced MyD88$^{-/-}$ mice with or without Nec-1. The extent of necrosis was obtained by histological examination. Lactate dehydrogenase (LDH) was also tested to examine the severity of necrosis. The results showed that cerulein-induced acinar cell necrosis was significantly reduced by the additional Nec-1 treatment in the MyD88$^{-/-}$ mice (*Figure* 7).

Effect of MyD88 deletion on L-arginine induced AP

To confirm that the enhanced effects of the MyD88$^{-/-}$ mice were not cerulein-induced AP model-specific, a L-arginine induced AP model was examined. The morphological changes were more severe in the MyD88$^{-/-}$ mice than the wild-type mice (*Figure 8*). Further, there were significantly higher levels of serum amylase (*Figure 9A*) and lipase

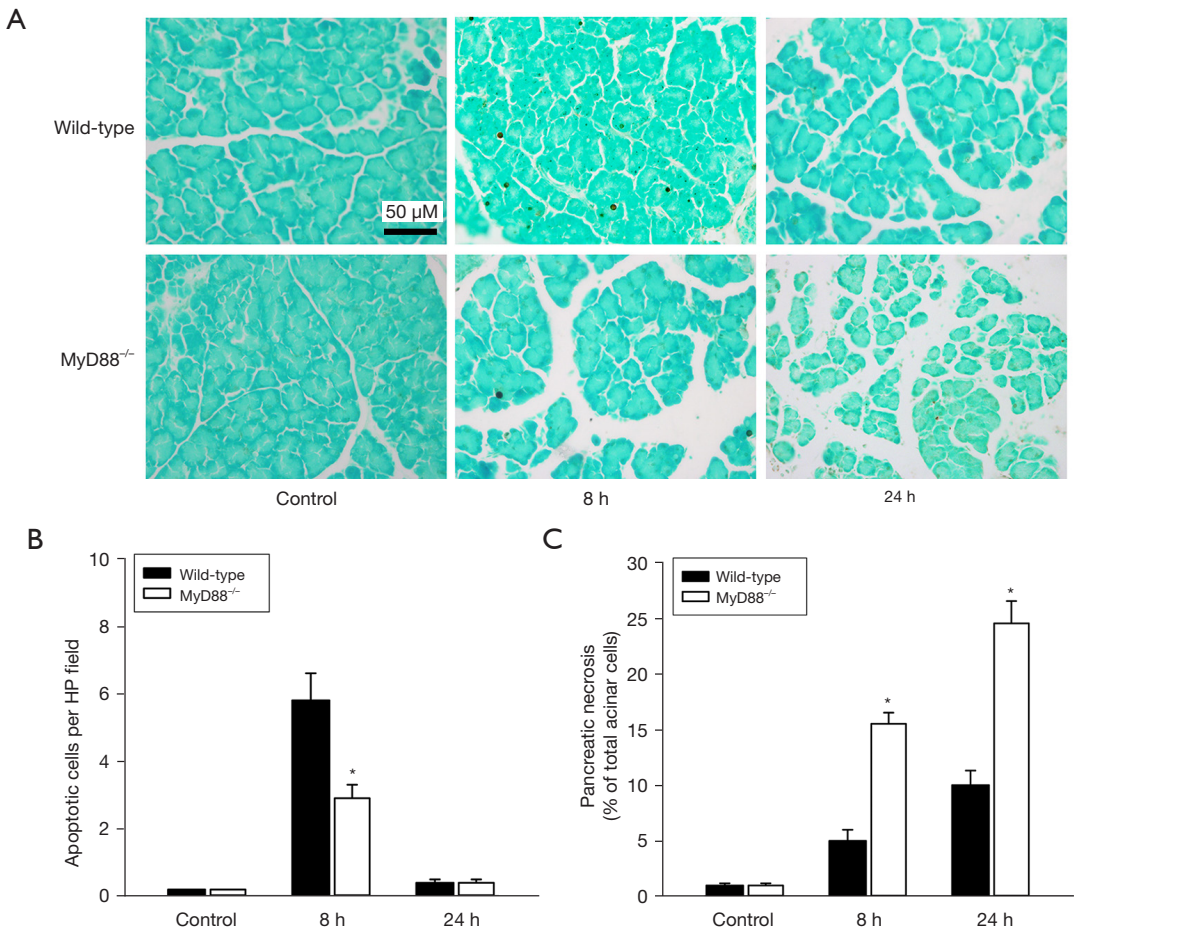

Figure 5 Effects of MyD88 deletion on cell death during cerulein-induced pancreatitis. Apoptosis was determined by TUNEL assays; representative images of a TUNEL-stained pancreas are shown (A). The results revealed a reduction in the number of apoptotic cells (B) and a significant increase in necrosis (C) in the MyD88$^{-/-}$ mice compared to the wild-type mice. The data are shown as the mean ± SEM. *, P<0.05, *vs.* wild-type. MyD88, myeloid differentiation primary response protein 88; SEM, standard error of the mean.

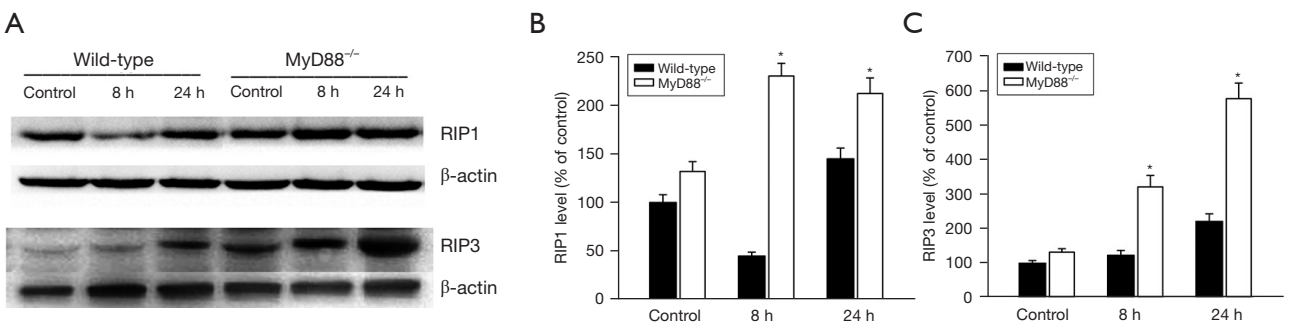

Figure 6 Effects of MyD88 deletion on necrotic mediators RIP1 and RIP3 expression in cerulein-induced pancreatitis. Western blots (A) were performed to measure the protein expression of RIP1 and RIP3. Quantification of RIP1 (B) and RIP3 (C) expression are shown. The data are shown as the mean ± SEM. *, P<0.05, *vs.* wild-type. MyD88, myeloid differentiation primary response protein 88; RIP1, receptor-interacting protein kinase 1; RIP3, receptor-interacting protein kinase 3; SEM, standard error of the mean.

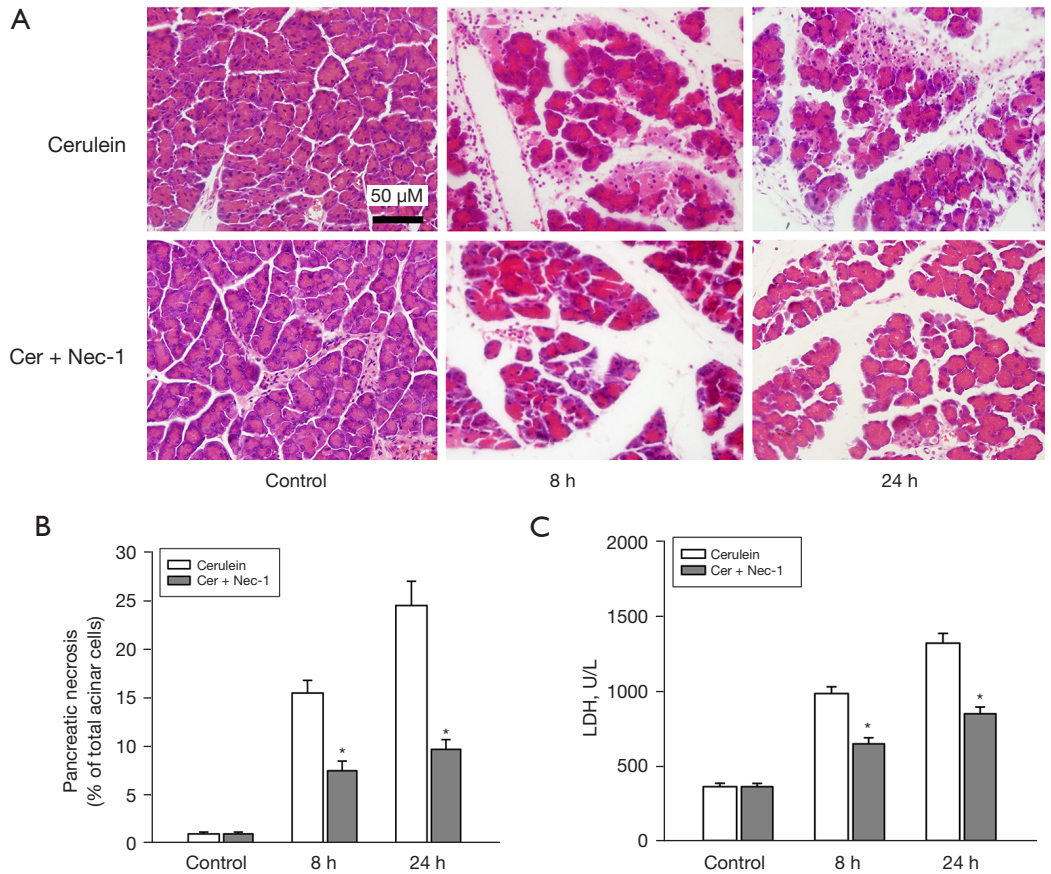

Figure 7 Effects of Nec-1 on cerulein-induced necrosis in MyD88$^{-/-}$ mice. The morphological changes were measured by H&E staining examinations (A). AP was induced by cerulein in the MyD88$^{-/-}$ mice. The control mice were injected with normal saline. Nec-1 (1.65 mg/kg) was injected intraperitoneally 10 min before the cerulein was injected. One-fourth of the above-mentioned concentration was administered to the MyD88$^{-/-}$ mice every 2 h later over a period of 6 h in total. The results demonstrated a significant reduction in necrosis (B) and LDH (C) in the Nec-1-treated mice compared to the cerulein-induced MyD88$^{-/-}$ mice. *, P<0.05, *vs.* cerulein-induced MyD88$^{-/-}$ mice. Nec-1, necrostatin-1; MyD88, myeloid differentiation primary response protein 88; H&E, hematoxylin and eosin; AP, acute pancreatitis; LDH, lactate dehydrogenase.

(*Figure 9B*), more pancreatic acinar cell necrosis (*Figure 9C*), and higher levels of LDH (*Figure 9D*) in the MyD88$^{-/-}$ mice than the wild-type mice.

Discussion

Inflammation and parenchymal cell death are characteristics of AP development (3). Once the premature intra-acinar digestive zymogens are activated, the injured acinar cells produce and release inflammatory mediators, and inflammatory cells are then recruited into the pancreas, which leads to systemic inflammation (24,25). TLRs are thought to be important receptors of the innate immune response. A previous study showed that TLR4, a typical TLR, plays a pro-inflammatory role in AP (12). MyD88 is the first and most essential utilized adaptor of TLR4 (26). The expression of Myd88 increases in AP pancreatic tissue (27). Previous studies have shown that Myd88 could affect the severity of AP. And Myd88 could mediate AP inflammation and SAP-related intestinal injury regulation (28,29). In addition, autophagy and oxidative stress play important roles in the pathogenesis of AP. MyD88 deficiency ameliorates oxidative injury in an autophagy-dependent mechanism (30). However, its role in AP remains unclear.

In the present study, MyD88-deficient mice were used to

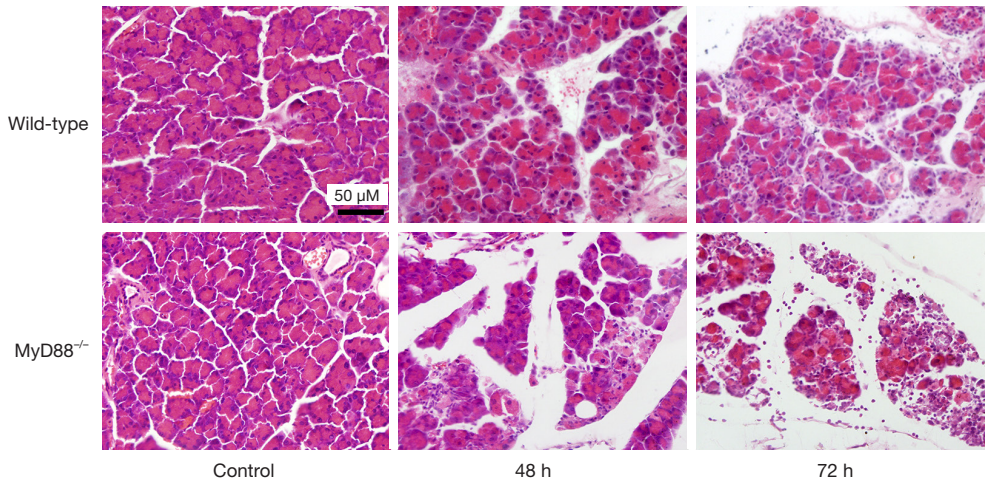

Figure 8 Effects of MyD88 deletion on the morphological changes in L-arginine induced pancreatitis. Representative micrographs of H&E-stained pancreas. The results revealed that the MyD88$^{-/-}$ mice had a significant increase in necrosis and inflammation compared to the wild-type mice. Saline was injected into the mice in the control groups. MyD88, myeloid differentiation primary response protein 88; H&E, hematoxylin and eosin.

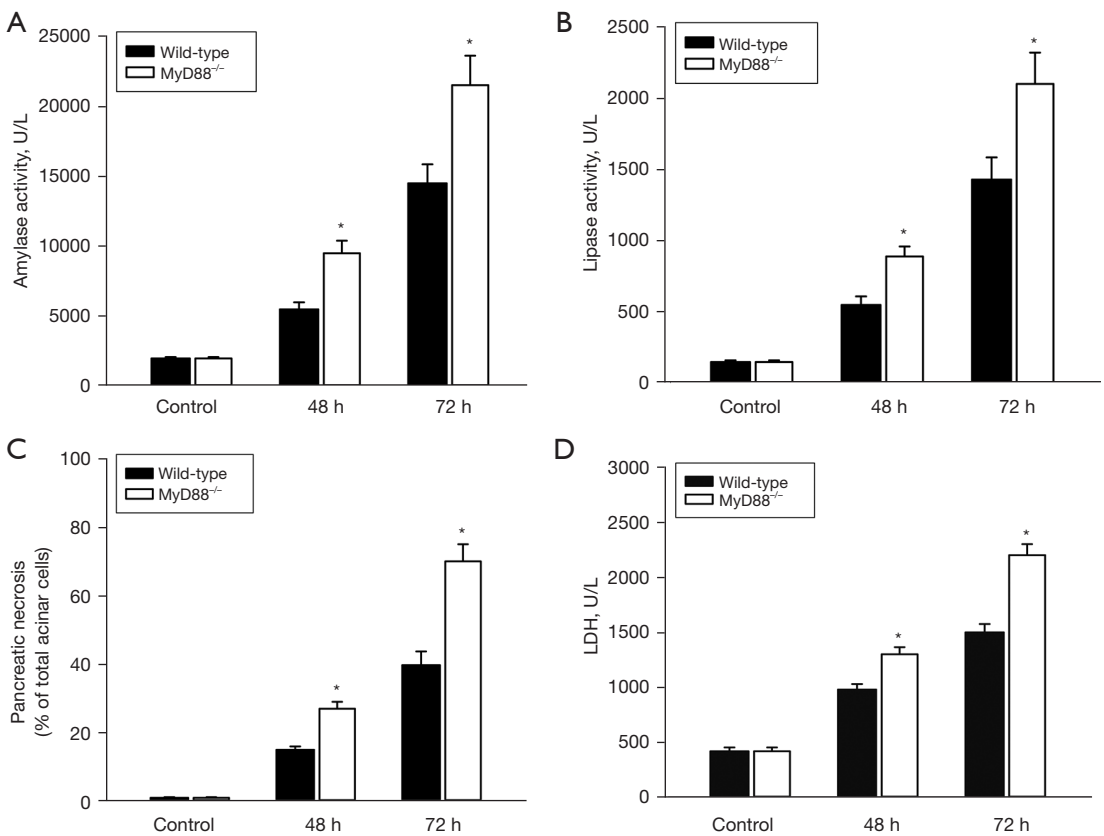

Figure 9 Effects of MyD88 deletion on the severity of L-arginine induced pancreatitis. The serum amylase (A), lipase (B), pancreatic necrosis (C), and LDH (D) levels of the MyD88$^{-/-}$ mice were significantly higher than those of the wild-type mice. The data are shown as the mean ± SEM. *, P<0.05, *vs.* wild-type. MyD88, myeloid differentiation primary response protein 88; LDH, lactate dehydrogenase; SEM, standard error of the mean.

establish a cerulein-induced AP model. The results showed that the severity of AP was increased in the MyD88$^{-/-}$ mice, which had increased levels of serum amylase and lipase, pancreatic MPO activity, and increased pancreatic acinar cell necrosis compared to the wild-type mice. Our findings that the MyD88$^{-/-}$ mice were likely to develop more severe pancreatitis may be surprising, as previous studies have reported that (12,13). As a downstream molecule of the TLR4 pathway, MyD88 should act concurrently with its upper stream regulator. To explain this unexpected finding, we measured the expression of TLR4, and found that the expression of TLR4 was significantly more enhanced in the MyD88$^{-/-}$ mice than the wild-type mice. Thus, we hypothesized that MyD88 was not the only pathway that acts with TLR4, and the other pathway may exert the opposite function in the progression of pancreatitis.

We then focused on both the MyD88-dependent and MyD88-independent TRIF signaling pathways. The MyD88-dependent signaling pathway was inhibited, which resulted in the inhibition of IL-10 in the MyD88$^{-/-}$ mice. IL-10 is the most potent anti-inflammatory cytokine, and research has shown that IL-10 protects the pancreas by stopping monocytes and macrophages from releasing pro-inflammatory factors and regulating systemic immune reactions and inflammatory reactions (31). Thus, the induction of IL-10 mediated by the MyD88-dependent signaling pathway is a protective immune defense mechanism that occurs in response to pancreatic tissue damage. Conversely, TRIF expression was significantly more increased in the MyD88$^{-/-}$ mice than the wild-type mice, indicating that the TRIF signaling pathway was primarily activated in the absence of MyD88. TRIF can mediate NF-κB and MAPK activation (16), resulting in the release of inflammatory cytokines at the late stage of this disease. In our study, the level of the pro-inflammatory cytokine, IL-6, was significantly increased at the late stage in the MyD88$^{-/-}$ mice. In addition, increased IL-6 levels have been shown to be proportional to the increased severity of AP (32).

TRIF plays an important role in many diseases. It could mediate production protective natural tumor-reactive IgM by B1 cells (33). In addition, preventive effect of lycopene in ulcerative colitis mice also through the TLR4-TRIF Pathway (34). Microglia necroptosis was regulated by TLR4-TRIF Pathway (35). In AP, loss of TRIF accelerates AP. And the TRIF depletion favors acinar cell necrosis to apoptosis (36). Thus, TRIF could affect the severity of AP. The TRIF signaling pathway also has been known to be involved in cell death signaling pathways, as TRIF has a RIP homotypic interaction motif that can physically interact with death receptors RIP1 and RIP3 (9,17). Apoptosis is considered predominantly protective (4), while necrosis can lead to organ damage and death (37). Apoptosis is mediated by caspase activation. Caspase also protects the pancreas from necrosis (4,38). The MyD88-dependent pathway has been reported to induce apoptosis through the TNF-α mediated caspase-8 pathway (39,40). Thus, the deletion of MyD88 led to less NF-κB activation and TNF-α production, all of which resulted in the reduced induction of caspase 8-mediated apoptosis.

Consistent with previous research, we found that apoptosis was lower in the MyD88$^{-/-}$ mice than the wild-type mice during cerulein-induced AP. Similarly, the level of TNF-α was more reduced in the MyD88$^{-/-}$ mice than the wild-type mice. The uncontrollable process was previously thought to be a characteristic of necrosis. However, recent research has shown that necrosis may occur as a controlled behavior (41). This so-called necroptosis is mediated by the 2 death receptors, RIP1 and RIP3 (9). Several recent studies have reported that TLRs activate programmed necrosis through the TRIF-RIP3 pathway or RIP1-RIP3 necrotic pathway (9,42).

In this study, both RIP1 and RIP3 expressions were more enhanced in the MyD88$^{-/-}$ mice than the wild-type mice. As a result, necrosis was significantly increased in the pancreas of the MyD88$^{-/-}$ mice. In addition, the special inhibitor of RIP1 Nec-1 reduced cerulein-induced necrosis in the MyD88$^{-/-}$ mice, but some necrosis was still observed. This finding indicates that the special inhibition of RIP1 decreased necrosis during cerulein-induced AP in MyD88$^{-/-}$ mice. The residue of necrosis might be due to the RIP1-independent RIP3-mediated necrotic pathway. A previous study also observed that RIP3 knockout mice showed less tissue damage in cerulein-induced AP than wild-type mice (43). Overall, these findings suggest that the deletion of MyD88 promotes necrosis and aggravates AP by enhancing the TRIF-mediated RIP1/RIP3 necrotic pathway.

A L-arginine induced AP model was adopted to confirm that the findings of the present study were not unique to the cerulean-induced AP model. The L-arginine-induced AP model was relatively more severe than the hyperstimulation cerulein model. It was also associated with a longer period (peak injury occurred in 72 h) than the cerulein model (44). In the present study, the necrosis of L-arginine-induced AP mice was significantly greater than that of the cerulein-induced AP mice. Deleting MyD88 also significantly

enhanced necrosis in the pancreas and aggravated the severity of L-arginine induced AP. These results proved that MyD88 is a significant determinant of the severity of AP, and its effect is not model-specific but common to all models tested.

Conclusions

The present study showed that MyD88 acts as a key mediator of the TLR4-mediated immune defense response and cell death in AP through the MyD88-dependent/MyD88-independent pathway. The deletion of MyD88 in AP decreased the induction of IL-10 and TNF-α mediated apoptosis, and enhanced MyD88-independent TRIF pathway-mediated necrosis via the activation of RIP1/RIP3, all of which led to increased necrosis and inflammation in the pancreas, which in turn aggravated the severity of AP. Thus, a lack of MyD88 inhibited the TLR4-mediated protective immune defense response and enhanced the MyD88-independent TRIF pathway, surprisingly accelerating the severity of AP largely by promoting pancreatic necrosis via the TRIF-mediated necrotic signaling pathway. The critical role of MyD88 in the immune defense response and cell death indicates that MyD88 represents a potential therapeutic target in the management of AP.

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Footnote

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